4500
FS-SO-2203-1.40
(Problem 2)

September 1977
Study Plan Summary
J. R. Bridges - Alexandria, La.

DOES NITROGEN FIXATION OCCUR IN BARK BEETLES?

In this study, the acetylene reduction assay will be used to determine whether nitrogen-fixing bacteria are associated with bark beetles and whether nitrogen fixation might be of significance to beetle growth and development. The possibility that symbionts of bark beetles are able to fix atmospheric nitrogen has been suggested as an explanation of the increase in total nitrogen concentration of pine inner bark that occurs folowing colonization of pine trees by the southern pine beetle, *Dendroctonus frontalis* Zimm., and its symbiotic microorganism complex.

UNITED STATES DEPARTMENT OF AGRICULTURE

FOREST SERVICE

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STUDY PLAN

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By

J. R. Bridges

RWU 2203

Southern Forest Experiment Station

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Prepared by:

Robert Bridges 9/19/77

J. R. Bridges
Research Entomolotist
RWU-SO-2203

Date

Approved by:

Peter L. Lorio, Jr. 9/23/77

Peter L. Lorio, Jr.
Project Leader
RWU-SO-2203

Date

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Nitrogen fixation by the symbionts of some insects, including bark beetles, was suggested many years ago (Peklo and Satava, 1949a and b; Csaky and Toth, 1948). However, unequivocal experimental evidence for these early reports was lacking and has been discounted (Benemann, 1973; French *et al.*, 1976). The development of the acetylene reduction assay for nitrogen fixation has made it possible to reinvestigate this problem. The assay is very sensitive and reliable and depends on the ability of nitrogenase to reduce acetylene to ethylene. Using this assay, nitrogen fixation has now been confirmed in several species of termites (Benemann, 1973; Breznak *et al.*, 1973; French *et al.*, 1976; Potrikus and Breznak, (1977). Citernesi *et al.*, (1977) also reported nitrogen fixation in the gastrointestinal tracts of 2 other insect species. Nitrogen fixation has not yet been unequivocally demonstrated in bark beetles.

In the past few years, nitrogen-fixing bacteria have been isolated from a number of diverse habitats. Apart from soil and legumes where nitrogen fixation is well documented (Benemann and Valentine, 1972), nitrogen fixing bacteria also occur in the intestines of man and animals (Bergensen and Hipsley, 1970), surface waters (Neilson and Sparell, 1976; Knowles *et al.*, 1974), the ocean (Werner *et al.*, 1973), the gastrointestine of ruminants (Hobson *et al.*), ruminants (Hobson *et al.*), the leaf surfaces of the Douglas fir (Jones, 1970), and decaying fir trees (Aho *et al.*, 1974).

The possibility that symbionts of bark beetles might be able to fix atmospheric nitrogen was suggested by Hodges *et al.*, (1968) as an explanation of their finding that the southern pine beetle (SPB), *Dendroctonus frontalis* Zimm., and its microorganism complex cause an increase in the total nitrogen concentration of pine inner bark. In view of this finding and the widespread occurrence of nitrogen-fixing bacteria in nature it seems worthwhile to investigate the possibility that nitrogen fixation might be associated with the SPB and other bark beetles.

OBJECTIVE

The objective of this study is to determine whether nitrogen-fixing bacteria are associated with bark beetles and whether nitrogen fixation might be of any significance to bark beetle growth and development.

MATERIALS AND METHODS

Nitrogen fixation assay.--The acetylene reduction assay will be used to test for nitrogen fixation. The assay has been reviewed and described in detail by several researchers (Postgate, 1972; Schollhorn and Burris, 1976; Hardy *et al.*, 1973; Hardy *et al.*, 1968). The method depends on the ability of nitrogenase to reduce acetylene to ethylene. In general, the assay is performed as follows: The preparation to be assayed (bacterial cultures, insects, bark tissue, etc.) is enclosed in a gas-tight container equipped with a serum stopper or other means to allow gas sampling. Acetylene is added usually to 0.1 - 0.2 atm. After a period of incubation samples are removed and analyzed for ethylene. The production of ethylene constitutes a positive test for nitrogenase. Ethylene will be assayed in this study with a gas chromatograph equipped with a column packed with Porapak T. Acetylene will be obtained commercially or generated in the laboratory from calcium carbide.

Isolation of nitrogen-fixing bacteria.--Bark beetles of various stages, frass, and bark adjacent to beetle galleries will be crushed in a nitrogen-free or nitrogen-limiting liquid medium in 50-ml serum bottles. Isolation of bacteria will also be attempted from the gut, surface-sterilized mycangium, and other parts of the insect's body. The medium for the isolation of anaerobic and facultative anaerobic bacteria will be that of Neilson and Sparell (1976) or Hino and Wilson (1958). Aerobic bacteria will be isolated using the medium of Brown *et al.* (1962). To obtain anaerobic conditions the bottles will be flushed with nitrogen filtered through a 0.22 μ millipore filter to ensure sterility. Bottles will be capped with serum stoppers and incubated with shaking at 30°C. Once growth is apparent 4 ml of acetylene will be added to each bottle. After incubating with acetylene for 30 minutes to several days, samples will be removed with a gas-tight syringe and analyzed for ethylene. Cultures giving positive tests will be subcultured several times to confirm nitrogen fixation and subsequently plated on the isolation media solidified with 1.5% agar. Bacteria will be obtained in pure culture by repeated subculturing, and pure cultures will be tested again for nitrogen fixation. The isolated bacteria will be identified using the methods described by Ewing (1962) and Meynell and Meynell (1970) or outlined in Bergey's Manual (1974).

In-situ nitrogen fixation.--To determine whether nitrogen fixation occurs in or on beetles, bark beetles of various stages (about 50/test) will be placed in 10-ml serum bottles. Air will be replaced with a argon: oxygen mixture (4:1) containing 8-10 μmoles of acetylene. After incubating for 1/2-2 hours, gas samples will be removed and analyzed for ethylene.

To determine whether nitrogen fixation is associated with bark tissues, pieces of bark will be assayed in the same way as beetles. Naturally infested trees also will be similarly assayed by confining small bolts in large desiccator jars equipped with a serum stopper. A portion of the agron:oxygen mixture will be removed with a hand vacuum pump and replaced with acetylene. Samples will be removed periodically for ethylene analysis. In each case controls without acetylene will be run to correct for ethylene production in the absence of acetylene.

PRESENTATION OF RESULTS

A final progress report will be prepared when the study is terminated. Preliminary results will be presented at the annual meeting of the Entomological Society of America in December, 1977.

DATES OF ESTABLISHMENT AND COMPLETION

The study will begin in August 1977 and will be completed in 1 year.

PERSONNEL ASSIGNMENT

J. Robert Bridges	Research Entomologist
Thelma Perry	Lab. Tech.
Faye Guinn	Lab. Tech.

REFERENCES CITED

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BIOMETRICS REVIEW ROUTING

ROUTING

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2. RWU 2203

RESEARCH WORK UNIT

☒ STUDY PLAN ☐ MANUSCRIPT ☐ RESPONSE TO BIOMETRICS REVIEW ☐ _____ (OTHER)

FS-SO-22031.40 "Does nitrogen fixation occur in bark beetles?"

(STUDY NUMBER) (TITLE)

J Robert Bridges 9/16/77

(AUTHOR SIGNATURE) J. Robert Bridges (DATE)

REMARKS:

Wish to have biometrics waived.

SUBMITTED: Peter P. Lowe, Jr. 9/16/77

(PROJECT LEADER SIGNATURE) (DATE)

AD

☐ FORWARDED FOR BIOMETRICS REVIEW ☒ BIOMETRICS REVIEW WAIVED

D H Hamilton 9/20/77

(ASSISTANT DIRECTOR SIGNATURE) (DATE)

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INSTRUCTIONS

1. Project Leader must submit this form with each study plan. Also use this form when manuscripts are submitted for review.
2. Insert desired "Routing" in space at top of form.
3. Be sure to insert proper identification in the "Research Work Unit" block.
4. Completed copies of this form will be filed in the study file in the Assistant Director's and Project Leader's files.

ORIGINAL – RESEARCH WORK UNIT FILE